JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of Aflatoxins in High-Pigment Content Samples by Matrix Solid-Phase Dispersion and High-Performance Liquid Chromatography

YAN-YUN HU,† PING ZHENG,‡ ZHAO-XIANG ZHANG,† AND YOU-ZHAO HE*,†

Department of Chemistry, University of Science and Technology of China, Hefei, Anhui 230026, People's Republic of China, and Inspection and Quarantine Technical Center, Anhui Entry−Exit Inspection and Quarantine Bureau, Hefei, Anhui 230061, People's Republic of China

A fast, efficient, and cost-effective method was developed for the analysis of aflatoxins in farm commodities with high-pigment content, such as chili powder, green bean, and black sesame. The proposed method involved matrix solid-phase dispersion (MSPD) and high-performance liquid chromatography (HPLC)-fluorescence detection (FLD) with postcolumn electrochemical derivatization in a Kobra cell. The MSPD procedure combined the extraction with neutral alumina and pigment cleanup with graphitic carbon black (GCB) in a single step. The recoveries of aflatoxins ranged from 88% to 95% with the relative standard deviations (RSD) less than 6% ($n = 6$). The limits of detection (LODs) were 0.25 ng/g aflatoxin B_1, G_1, and 0.10 ng/g aflatoxin B_2, G_2, respectively. The analytical results obtained by MSPD were compared to those of the immunoaffinity column (IAC) cleanup method. No significant differences were found between the two methods by *t*-test at the 95% confidence level.

KEYWORDS: Aflatoxins; matrix solid-phase dispersion; high-performance liquid chromatography; high-pigment content sample

INTRODUCTION

Aflatoxins are the secondary metabolites of fungi (*Aspergillus flavus* and *A. parasiticus*) and can be found widely in food and feed. They are considered as the genetic damaging and carcinogenic compounds for human and animals (*1*). Because of the potential health risk, the contamination of aflatoxins in food has been regulated in many countries, and rigid maximum residue limits (MRL) for aflatoxins have been established (*2*, *3*).

Methods for the determination of aflatoxins include thin-layer chromatography (TLC) (*4*), high-performance liquid chromatography (HPLC) (*5*−*7*), and enzyme-linked immunosorbent assay (ELISA) (*8*, *9*). TLC is widely used for aflatoxin analysis because of its simplicity and practicability. However, it has the drawbacks of low sensitivity and poor accuracy. As a fast screening method, ELISA possesses good specificity, sensitivity, and simplicity. However, it has the possibility of false positives because of cross-reaction and interference in the complex matrixes. The present trend is the use of HPLC as the alternative technique for aflatoxin analysis due to its high accuracy and potential for automation.

Before aflatoxin determination, a sequence of sample pretreatment steps is needed. Among them, extraction and purification processes are the most difficult, but they are important steps that depend significantly on the physicochemical properties of the samples contaminated with aflatoxins. Sample with high pigment and lipid content makes the aflatoxin analysis difficult because of serious matrix interference. Hence, a more selective treatment followed by specific purification is required before the analysis. Some attempts have been made to purify aflatoxins, such as solvent extraction followed by liquid−liquid extraction (*10*) or solid-phase extraction (SPE). A variety of SPE columns were used for purification, including silica (*11*), C_{18} (*12*), Oasis HLB cartridge (*7*), and multifunctional columns (MFC) (*13*, *14*), etc. Immunoaffinity columns (IAC) were also adopted for the purification of aflatoxins (*5*, *15*, *16*), which are efficient, but rather expensive. Matrix solid-phase dispersion (MSPD) is an efficient method for sample pretreatment developed by Braker et al. (*17*). It combines the extraction and cleanup in a single step, thus reducing solvent consumption and sample treatment time (*18*). MSPD was applied to the analysis of aflatoxins in high fat content samples (*19*), in which C_{18} sorbent was used to remove lipid interference in peanut matrix. However, few studies were reported using MSPD for pigment cleanup in the analysis of aflatoxins in high-pigment content samples, such as chili powder, green bean, and black sesame.

In this study, a fast, efficient, and cost-effective method was developed for the aflatoxin analysis in high-pigment samples. Aflatoxin extraction and matrix cleanup were carried out in a single step by MSPD pretreatment with neutral alumina and

* Author to whom correspondence should be addressed [telephone +86 551 3607072; fax +86 551 3603388; e-mail yzhe@ustc.edu.cn].
† University of Science and Technology of China.
‡ Anhui Entry−Exit Inspection and Quarantine Bureau.

10.1021/jf0601564 CCC: $33.50
Published on Web 05/20/2006

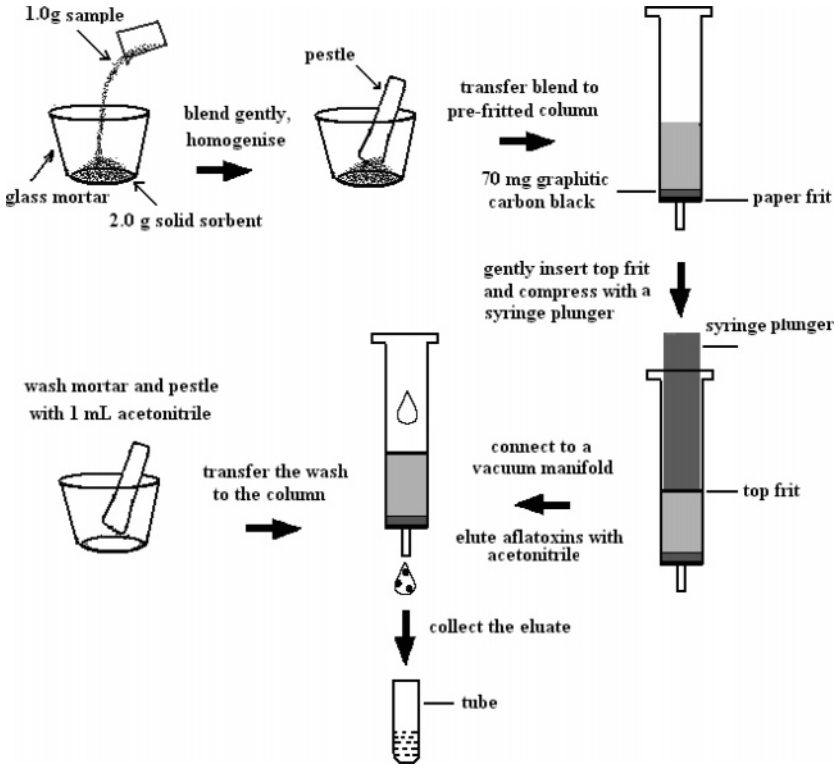

Figure 1. Schematic diagrams of the whole procedure in matrix solid-phase dispersion.

graphitic carbon black (GCB). The aflatoxins were separated and detected by HPLC-fluorescence detection (FLD) after the postcolumn electrochemical derivatization in a Kobra cell. The purification results obtained with MSPD were compared to those of IAC, in terms of the limit of detection (LOD) and aflatoxin recovery.

MATERIALS AND METHODS

Reagents and Solutions. The mixed standard reagent of aflatoxins was purchased from Supelco (Bellefonte, USA) with the concentrations of 1.0 μg/mL aflatoxin B_1, G_1, and 0.3 μg/mL aflatoxin B_2, G_2. The stock solution was prepared by diluting the standard reagent with methanol and was stored in a lightproof container at −20 °C. The stock solution could be used for 3 months. The working solutions were obtained by diluting the stock solution further with methanol covering the concentration range of 0.5−10 ng/mL for aflatoxin B_1, G_1 and 0.15−3.0 ng/mL for aflatoxin B_2, G_2.

All of the solvents were of chromatographic grade, and distilled water through a 0.45-μm-filter membrane was used. Acetonitrile, acetone, methanol, and methylene chloride were purchased from Merck (Darmstadt, Germany). The solutions were filtrated through 0.45-μm filter membrane and degassed for 30 min by an ultrasonic bath before use. Florisil PR (60−100 mesh) from Fluka (Buchs, Switzerland), octadecylsilica (50 μm), silica gel 60 (230−400 mesh), and neutral alumina (60−100 mesh) from Merck were tested as solid adsorbents for MSPD pretreatment. GCB (120−400 mesh, 100 m^2/g) from Supelco was used as cleanup material. All of the sorbents were not deactivated. Aflaprep immunoaffinity columns were obtained from Vicam (Watertown, USA).

Apparatus. The HPLC separation was carried out by an Agilent 1100 liquid chromatography system with a 250 × 4.6 mm i.d, 5 μm Zorbax SB C_{18} reversed-phase column and a 20 × 4.6 mm i.d, 5 μm HP C_{18} precolumn (Agilent, USA). The sample injection volume was 20 μL. The mobile phase was the mixed solution of methanol−acetonitrile−water (2/2/6, v/v/v) containing 0.12 g/L potassium bromide and 200 μL/L nitric acid, and its flow rate was 1.0 mL/min. In the reversed HPLC system, the fluorescence of aflatoxin B_1 and G_1 was rather weak (*20*). To enhance the fluorescent responses of aflatoxin B_1 and G_1, an on-line and postcolumn derivatization was carried out with bromine generated in a Kobra cell (Rhone Diagnostics, Glasgow, U.K.) with the electrochemical reaction current of 100 μA. The postcolumn reaction coil consists of a 360 mm × 0.8 mm i.d. PTFE tubing. Aflatoxins were detected with a scanning fluorescence detector at the excitation and emission wavelengths of 355 and 435 nm, respectively.

MSPD Procedure. The whole setup and procedure of MSPD was illustrated in **Figure 1**. First, samples were milled in a food chopper, and 1.00 g of treated sample was transferred to a glass mortar containing 2.00 g of solid adsorbents. For the spiked sample, the standard aflatoxin solution was added into the sample. The sample was blended gently with sorbents by a pestle. The homogeneous mixture was then transferred into the glass cartridge (MSPD column) containing 70 mg of GCB in the bottom and underlaid with a glass microfiber filter paper (Whatman GF/A). The sample mixture was covered with another filter paper and lightly compressed with a modified syringe plunger to form a MSPD column. It was then connected to a vacuum manifold. The mortar and pestle were washed with 1 mL of acetonitrile, and the wash was transferred into the column. Another 4 mL of acetonitrile was added, and the column was eluted at a flow rate of 2 mL/min. All of the eluate was collected and concentrated to dryness under N_2 flow in a water bath at 40 °C, and then dissolved in 0.5 mL of acetonitrile for HPLC analysis.

The extraction procedure described above is based on the data obtained from different optimization assays. They involved the study of different solid sorbents for MSPD including octadecylsilica, Florisil, silica gel,and neutral alumina, and also different elution volume. Moreover, the optimization of the procedure included an assessment of an additional purification step with GCB.

IAC Cleanup. The homogeneous samples of 5.00 g were extracted with 20 mL of 80% methanol (v/v) by vortexing with a MS1 Minishaker (IKA Works Inc., Guangzhou, China) at 2500 rpm for 2 min. The extracted mixture was then filtered through Whatman No.3 filter paper, and 4 mL of the filtered solution was diluted to 20 mL with distilled water. After that, the solution was passed through the immunoaffinity column at a flow rate of 2 mL/min. The column was subsequently washed with 10 mL of distilled water and then purged to dryness with air. The aflatoxins were eluted dropwise with 2 mL of methanol. The eluate was then concentrated to 0.5 mL by N_2 flow for HPLC analysis.

Recovery Experiment. Recovery experiment was performed with spiked sample at the concentration level of 2.5 ng/g aflatoxin B_1, G_1 and 0.75 ng/g aflatoxin B_2, G_2. The spiked and unspiked samples were

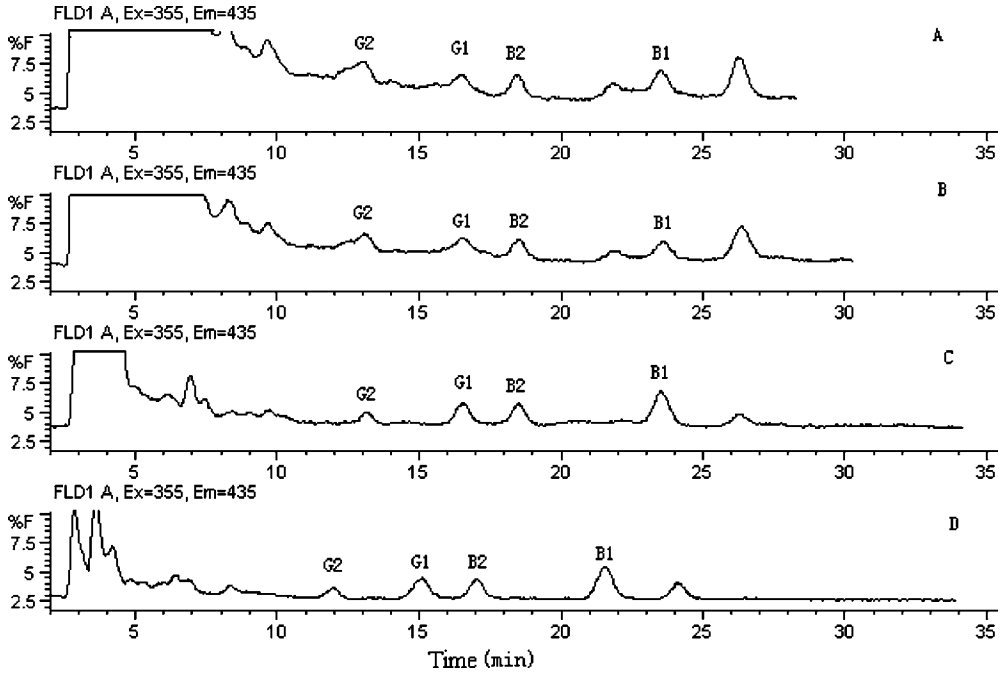

Figure 2. Chromatograms for analysis of aflatoxins in chili powder obtained from (**A**) ODS (C_{18}), (**B**) silica, (**C**) Florisil, and (**D**) neutral alumina.

extracted and cleaned up according to the method described above. The recovery of aflatoxins in each sample was calculated by subtraction of any aflatoxins detected in the unspiked samples. The analytical precision was evaluated with relative standard derivation (RSD) by six-replicated analysis of the samples.

Quantitation and Identification. The quantitative analysis was carried out by peak area using the external standard method. The calibration curves and linear regression equations were obtained with five mixed standard solutions covering the concentration range of 0.5−10 ng/mL for aflatoxin B_1, G_1, and 0.15−3.0 ng/mL for aflatoxin B_2, G_2. Identification of aflatoxins was based on retention time. A mixed standard solution of aflatoxins was subsequently separated after the sample analysis for the exact identification every day.

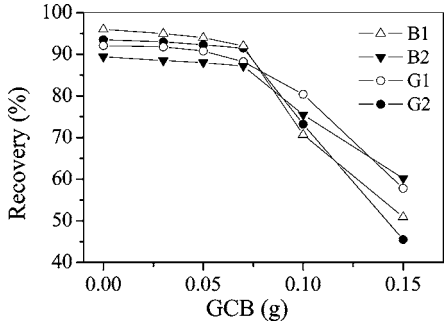

Figure 3. Effect of graphitic carbon black amount on the recoveries of aflatoxins for 1.0 g of chili powder spiked with 2.5 ng/g aflatoxin B_1 and G_1, and 0.75 ng/g aflatoxin B_2 and G_2.

RESULTS AND DISCUSSION

Selection of Solid Sorbent. The effect of different sorbents on aflatoxin recovery and matrix cleanup degree was studied for MSPD, including C_{18}-silica, silica gel, Florisil, and neutral alumina. Here, the unspiked and spiked chili powder samples were used to optimize the MSPD conditions because of its more complex matrixes than others. The spiking levels in chili powder were 2.5 ng/g aflatoxin B_1, G_1, and 0.75 ng/g aflatoxin B_2, G_2. The experimental results indicated that these sorbents have similar recoveries for aflatoxin B_1, B_2, G_1, and G_2, but quite different capacity in the matrix cleanup for chili powder. The chromatograms obtained with four sorbents are illustrated in **Figure 2**. The sample treated by C_{18}-silica gave the highest background, while three polar sorbents, that is, silica, Florisil, and neutral alumina, produced lower background than C_{18}-silica. Among them, neutral alumina was the most effective in removing the interferences, as evidenced by the lowest background level in the chromatograms. The high background might be caused by chili pigments and capsaicinoids in the sample extract, which are the common components in chili powder. The polar capsaicinoids were poorly retained on the hydrophobic C_{18}-silica sorbent and easily eluted by acetonitrile solvent. However, neutral alumina proved to be a better dispersant than the other sorbents assessed due to its hydrophilic characteristics, which provided high affinity for polar compounds. Because the sample extract obtained with C_{18}-silica, Florisil, and silica showed many interfering peaks, neutral alumina was selected for MSPD. However, due to the presence of different polar pigments in chili powder such as capsanthin, capsorubin, zeaxanthin, and cryptoxanthin, individual sorbent had difficulty eliminating various pigments efficiently. Although the pigments seemed to have a slight effect on chromatographic separation, an additional purification is also required due to the potential contamination and deterioration of the injection system and separation column.

Purification. For the pigment removal, carbon-based sorbent was reported to be efficient due to various functional groups on the surface (*21*). In this work, GCB sorbent was used for co-column cleanup and packed into the bottom of the same column as the MSPD material. Because GCB has large adsorption capacity not only for pigments, but also for aflatoxins, GCB cleanup carries the risk of low recovery for aflatoxins. However, due to the complex surface properties, GCB can preferentially adsorb macromolecules, such as pigments and lipids. Once the GCB is saturated by macromolecule adsorption, it cannot adsorb aflatoxins. Hence, the amount of GCB has an important influence on the recovery of aflatoxins and should be rigidly optimized according to the sample amount. **Figure 3** illustrates the effect of the GCB amount on the recoveries of aflatoxins for the purification of 1.0 g of chili powder spiked with 2.5 ng/g aflatoxin G_1, B_1, and 0.75 ng/g aflatoxin B_2, G_2.

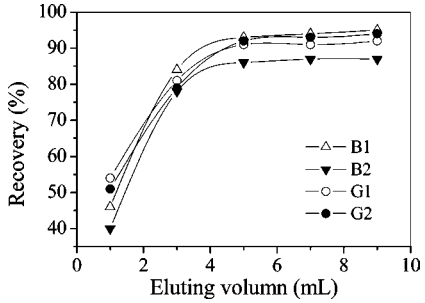

Figure 4. Effect of elution volume on recoveries of aflatoxins for 1.0 g of chili powder spiked with 2.5 ng/g aflatoxin B_1 and G_1, and 0.75 ng/g aflatoxin B_2 and G_2.

Table 1. Comparison of Analytical Results Obtained by MSPD and IAC Methods

	mean recoveries (%) +RSD (%) ($n = 6$)							
	chili powder		black sesame		green bean		LOD ng g^{-1}	
aflatoxin	MSPD	IAC	MSPD	IAC	MSPD	IAC	MSPD	IAC
B1	95 ± 3	92 ± 2	94 ± 2	91 ± 2	92 ± 3	94 ± 3	0.25	0.20
B2	88 ± 5	93 ± 3	90 ± 3	91 ± 2	91 ± 2	93 ± 2	0.10	0.10
G1	92 ± 4	91 ± 2	90 ± 3	86 ± 4	90 ± 4	92 ± 2	0.25	0.20
G2	93 ± 6	91 ± 4	90 ± 4	90 ± 3	89 ± 3	90 ± 4	0.10	0.10

The results showed that the increase of GCB amount caused the decrease of aflatoxin recoveries. A higher amount of GCB possessed good capacity for removing pigment and gave cleaner extracts, but the recoveries of aflatoxins B_1, B_2, G_1, and G_2 were unsatisfactory. Furthermore, aflatoxins could not be quantitatively eluted from the GCB column even by the different eluting solvents, including methanol, dichoromethane, acetone, and various proportions of dicholomethane−acetonitrile mixture. This demonstrated that excess GCB led to irreversible adsorption for aflatoxins. On the other hand, a lower amount of GCB gave poor capacity for pigment cleanup. Here, 70 mg of GCB was chosen as the compromise between aflatoxin recoveries and pigment purification.

The effect of elution volume on aflatoxin recoveries is shown in **Figure 4**. The recovery of each aflatoxin increased rapidly to nearly 90% by increasing the acetonitrile volume to 5 mL, and then reached an equilibrium value with increasing the elution volume further. Therefore, the eluting volume of 5 mL of acetonitrile was chosen in this work.

Comparison of MSPD and IAC Pretreatment for Aflatoxin Analysis. In this study, the MSPD method coupled with neutral alumina extraction and GCB cleanup was also used to analyze other high-pigment content samples of black sesame and green bean besides chili powder. The analytical performance of MSPD was evaluated and compared to the IAC method. Black sesame, green bean, and chili powder were spiked with 2.5 ng/g aflatoxin B_1, G_1, and 0.75 ng/g aflatoxin B_2, G_2, respectively. The average recoveries of aflatoxins and LODs obtained by the two methods are listed in **Table 1**. Here, LOD is defined as the aflatoxin concentration of 3 times the signal-to-noise ratio. The average recoveries and LODs of aflatoxins obtained from MSPD and IAC were statistically evaluated by a *t*-test at the confidence level of 95%. No significant differences were observed between the two methods. Using MSPD, the average recoveries of aflatoxins were higher than 88% with RSD less than 6% in different sample matrixes, and LODs were 0.25 ng/g for aflatoxins B_1, G_1, and 0.10 ng/g for aflatoxins B_2, G_2, respectively. The cleanup degree of the two methods could be evaluated from the chromatograms, as shown in **Figure 5**. It

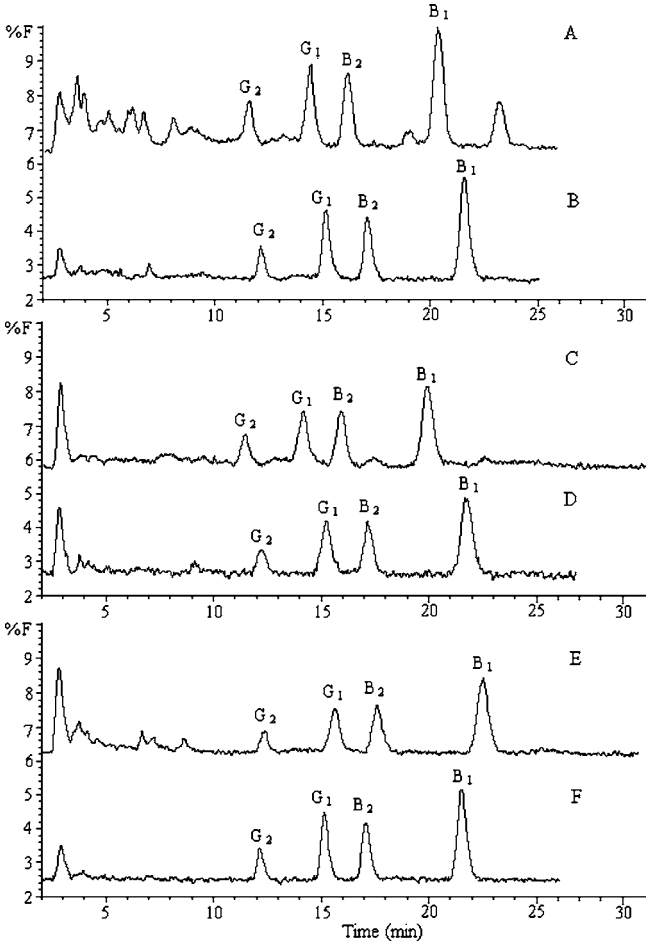

Figure 5. Comparison of chromatograms obtained with matrix solid-phase dispersion extraction (**A**, **C**, **E**) and immunoaffinity column (**B**, **D**, **F**) cleanup for analysis of aflatoxins in chili powder (**A**, **B**), black sesame (**C**, **D**), and green bean (**E**, **F**).

demonstrated that the chromatograms of black sesame and green bean by MSPD were quite similar to those by IAC. Although the chromatogram of chili powder had a few impurity peaks by MSPD, it did not disturb the separation peaks of aflatoxins. The total pretreatment time and solvent volume required for one sample analysis were less than 20 min and 5 mL, respectively. For the samples investigated in this study, the MSPD method was as accurate and precise as IAC, but more preferable in solvent consumption and experimental cost.

Analysis of Real Samples. Aflatoxin contamination has been found in foodstuff, including chili powder, sesame (*22*), and bean samples (*23*). Usually, aflatoxin analysis in these samples is required in the commercial transactions for minimizing the public health risk. In this study, 20 samples obtained from different supermarkets, including chili powder, black sesame, and green bean, were analyzed with the proposed method. Aflatoxins B_1, B_2, G_1, and G_2 were not detected in these samples. To validate the results obtained with the MSPD method, IAC pretreatment was also employed in the sample analysis. The obtained results with IAC were consistent with those of MSPD, as shown in the chromatograms of **Figure 6**. Because MSPD based on neutral alumina extraction and GCB cleanup is efficient, accurate, and cost-effective, its application in the aflatoxin analysis in high-pigment content samples can be recommended.

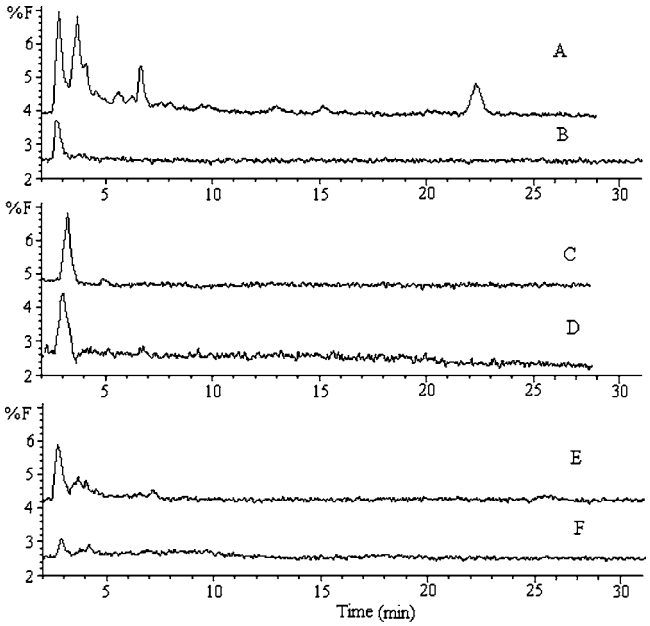

Figure 6. Chromatograms of real samples obtained with matrix solid-phase dispersion extraction (**A**, **C**, **E**) and immunoaffinity column (**B**, **D**, **F**) cleanup for analysis of aflatoxins in chili powder (**A**, **B**), black sesame (**C**, **D**), and green bean (**E**, **F**).

LITERATURE CITED

(1) Moss, M. O. Risk assessment for aflatoxin in foodstuffs. *Int. Biodeterior. Biodegrad.* **2002**, *50*, 137−142.

(2) Ismail, Y. S. Rustom Aflatoxin in food and feed: occurrence, legislation and inactivation by physical methods. *Food Chem.* **1997**, *59*, 57−67.

(3) Commission Regulation (EC) NO 466/2001 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs. *Off. J. Eur. Communities* **2001**, *L71*, 1−13.

(4) Lin, L. M.; Zhang, J.; Wang, P.; Wang, Y. S.; Chen, J. P. Thin-layer chromatography of mycotoxins and comparison with other chromatographic methods. *J. Chromatogr., A* **1998**, *815*, 3−20.

(5) Garcia-Villanova, R. J.; Cordon, C.; Paramas, A. M. G.; Aparicio, P.; Rosales, M. E. G. Simultaneous immunoaffinity column cleanup and HPLC analysis of aflatoxins and ochratoxin A in Spanish bee pollen. *J. Agric. Food Chem.* **2004**, *52*, 7235−7239.

(6) Ahmed, I. A.; Robinson, R. K. Selection of a suitable method for analysis of aflatoxins in date fruits. *J. Agric. Food Chem.* **1998**, *46*, 580−584.

(7) Ventura, M.; Gomez, A.; Anaya, I.; Diaz, J.; Broto, F.; Agut, M.; Comellas, L. Determination of aflatoxins B_1, G_1, B_2, and G_2 in medicinal herbs by liquid chromatography-tandem mass spectrometry. *J. Chromatogr., A* **2004**, *1048*, 25−29.

(8) Aycicek, H.; Aksoy, A.; Saygi, S. Determination of aflatoxin levels in some dairy and food products which consumed in Ankara, Turkey. *Food Contr.* **2005**, *16*, 263−266.

(9) Lipigorngoson, S.; Limtrakul, P.; Suttajit, M.; Yoshizawa, T. In-house direct cELISA for determining aflatoxin B-1 in Thai corn and peanuts. *Food Addit. Contam.* **2003**, *20*, 838−845.

(10) Barbieri, G.; Bergamini, C.; Ori, E.; Resca, P.; Aflatoxin (M1) in parmesan cheese-HPLC determination. *J. Food Sci.* **1994**, *59*, 1313 and 1331.

(11) Scholten, J. M.; Spanjer, M. C. Determination of aflatoxin B-1 in pistachio kernels and shells *J. AOAC Int.* **1996**, *79*, 1360−1364.

(12) Ferreira, I. M. P. L. V. O.; Mendes, E.; Oliveira, M. B. P. P. Quantification of aflatoxins B-1, B-2, G (1), and G (2) in pepper by HPLC/fluorescence. *J. Liq. Chromatogr. Relat. Technol.* **2004**, *27*, 325−334.

(13) Akiyama, H.; Chen, D. Y.; Miyahara, M.; Toyoda, M.; Saito, Y. Simple HPLC determination of aflatoxins B-1, B-2, G(1), and G(2) in nuts and corn. *J. Food Hyg. Soc. Jpn.* **1996**, *37*, 195−201.

(14) Akiyama, H.; Goda, Y.; Tanaka, T.; Toyoda, M. Determination of aflatoxins B1, B2, G1, and G2 in spices using a multifunctional column clean-up. *J. Chromatogr., A* **2001**, *932*, 153−157.

(15) Stroka, J.; van Otterdijk, R.; Anklam, E. Immunoaffinity column clean-up prior to thin-layer chromatography for the determination of aflatoxins in various food matrices. *J. Chromatogr., A* **2000**, *904*, 251−256.

(16) Kussak, A.; Andersson, B.; Andersson, K. Determination of aflatoxins in airborne dust from feed factories by automated immunoaffinity column cleanup and liquid chromatography. *J. Chromatogr., A* **1995**, *708*, 55−60.

(17) Barker, S. A.; Long, A. R.; Short, C. R. Isolation of drug residues from tissues by solid-phase dispersion. *J. Chromatogr.* **1989**, *475*, 353−361.

(18) Hu, Y. Y.; Zheng, P.; He, Y. Z.; Sheng, G. P. Response surface optimization for determination of pesticide multiresidues by matrix solid-phase dispersion and gas chromatography. *J. Chromatogr., A* **2005**, *1098*, 188−193.

(19) Blesa, J.; Soriano, J. M.; Molto, J. C.; Marin, R.; Manes, J. Determination of aflatoxins in peanuts by matrix solid-phase dispersion and liquid chromatography. *J. Chromatogr., A* **2003**, *1011*, 49−54.

(20) Kok, W. Th. Derivatization reactions for the determination of aflatoxins by liquid chromatography with fluorescence detection. *J. Chromatogr., B* **1994**, *652*, 127−137.

(21) Barriada-Pereira, M.; Gonzalez-Castro, M. J.; Muniategui-Lorenzo, S.; Lopez-Mahia, P.; Prada-Rodriguez, D.; Fernandez-Fernandez, E. Determination of 21 organochlorine pesticides in tree leaves using solid-phase extraction clean-up cartridges. *J. Chromatogr., A* **2004**, *1061*, 133−139.

(22) Tabata, S.; Kamimura, H.; Ibe, A.; Hashimoto, H.; Iida, M.; Tamura, Y.; Nishima, T. Aflatoxin contamination in foods and feedstuffs in Tokyo: 1986−1990. *J. AOAC Int.* **1993**, *76*, 32−35.

(23) Pinto, V. E. F.; Vaamonde, G.; Brizzio, S. B.; Apro, N. Aflatoxin production in soybean varieties grown in Argentina. *J. Food Prot.* **1991**, *54*, 542−545.

Received for review January 19, 2006. Revised manuscript received March 20, 2006. Accepted March 21, 2006. We thank the Scientific Foundation of the University (USTC) for the financial support to carry out this work.

JF0601564